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Original research article

Putative DNA-dependent RNA polymerase in Mitochondrial Plasmid of *Paramecium caudatum* Stock GT704

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ABSTRACT

Mitochondria of *Paramecium caudatum* stock GT704 has a set of four kinds of linear plasmids with sizes of 8.2, 4.1, 2.8 and 1.4 kb. The plasmids of 8.2 and 2.8 kb exist as dimers consisting of 4.1- and 1.4-kb monomers, respectively. The plasmid 2.8 kb, designated as pGT704-2.8, contains an open reading frame encodes for putative DNA-dependent RNA polymerase (RNAP). This study reveals that this RNAP belongs to superfamily of DNA/RNA polymerase and family of T7/T3 single chain RNA polymerase and those of mitochondrial plasmid of fungi belonging to Basidiomycota and Ascomycota. It is suggested that RNAP of pGT704-2.8 can perform transcription without transcription factor as promoter recognition. Given that only two motifs were found, it could not be ascertained whether this RNAP has a full function independently or integrated with mtDNA in carrying out its function.

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1. Introduction

Plasmids are extrachromosomal genetic elements found in various organisms. Two types of plasmid structures known so far, namely the double-stranded closed circular plasmid which commonly found in cells of prokaryotes and some eukaryotes, and single- and double-stranded linear plasmids found in fungi, yeast, and ciliates (Tallei *et al.* 2002; Hausner 2003).

Linear plasmid has terminal inverted repeat at both ends and covalently-bound terminal protein (TP) at 5' end which is similar to some viral genome structure (Andrade *et al.* 2009). Two open reading frames (ORFs) of these plasmids encode for DNA and RNA polymerases which are required for their replication (Griffiths 1995; Oeser and Tudzynski 1989; Chan *et al.* 1991). RNA polymerase (RNAP) in the linear plasmids are DNA-directed RNAP (DNA-dependent RNAP) and abbreviated as RNAP, which serves to catalyze the synthesis of a DNA chain using DNA as a template. The RNAP characteristic is similar to those of the bacteriophage and yeast mitochondria. The 5' end, besides being involved in replication, also has a role in the integration of the plasmid into mitochondrial genome (Griffiths 1995; Andrade *et al.* 2009; Andrade *et al.* 2013). Mitochondrial plasmid of *Moniliophthora perniciosa* can integrate fully into the mitochondrial genome (Andrade *et al.*

2009). The plasmid is likely to have a function in the aging process (senescence) (Chan *et al.* 1991; van Diepeningen *et al.* 2008).

Paramecium is the first example in which an mtplasmid was discovered among ciliates. *Paramecium caudatum* stock GT704 has type-II DNA with two sets of mtplasmids. The 8.2 and 2.8 kb DNAs are dimers of the 4.1 and 1.4 kb DNA, respectively (Tallei *et al.* 2002; Endoh *et al.* 1994). The plasmid 2.8 kb (designated as pGT704-2.8) has an ORF for RNAP. This study aimed at characterizing RNAP encoded by pGT704-2.8 and analyzing its relationship with RNAPs from other organisms to predict its function in mitochondria.

2. Material and Methods

2.1. Stock and culture

From previous research conducted by Tallei *et al.* (2002), *P. caudatum* stock GT704 used in this study contains plasmid type II. Plasmid type II always has four types of plasmid DNA with sizes of 8.2, 4.1, 2.8, and 1.4 kb. Plasmid DNA 8.2 and 2.8 kb are always in the form of dimer, each of monomer molecule of 4.1 and 1.4 kb, respectively. The cells of *P. caudatum* were grown at 25 °C in fresh lettuce juice medium inoculated with non-pathogenic strains of *Klebsiella pneumoniae* 1 day before use.

2.2. Purification of mitochondria

The cell suspension was filtered with gauze to remove dirt, centrifuged, and washed twice in a Dryl solution (Tsukii *et al.* 1994). Mitochondrial purification was performed following the method

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of [Goddard and Cummings \(1975\)](#) with slight modification. The centrifuged cells were resuspended in two times volume of ice cold mannitol solution (0.44 M mannitol, 1 mM KCl, 1 mM Tris-HCl pH 7.5, and 0.25% bovine serum albumin). Cells of *P. caudatum* were crushed using a homogenizer until almost all the cells ruptured. Homogenized cells were resuspended in ice cold mannitol and centrifuged at 600 g for 6 minutes at 0 °C. Supernatant was centrifuged at 5,000 g for 6 minutes. Mitochondrial pellets were resuspended in 1 mL of mannitol solution containing 30 U of DNase I (Takara Shuzo Co., Japan) and 5 mM MgCl₂. Mitochondrial suspension was incubated on ice for 25 minutes and added with mannitol solution containing 10 mM EDTA, followed by centrifugation at 5,000 g for 6 minutes. Pellets were used for pure mitochondrial fraction.

2.3. Extraction of mitochondrial DNA and plasmids

Mitochondria were lysed in 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, 1% (w/v) sodium dodecyl sulfate and 0.1 mg/mL proteinase K for 50 °C for 3 hours. Lysate was extracted twice with phenol (same volume) and treated with proteinase K ([Tsukii et al. 1994](#)).

2.4. DNA sequencing

Nucleotide sequencing of pGT-704-2.8 kb was conducted at Genetics Laboratory, University of Kanazawa, Japan.

3. Results

Mtplasmids 8.2 and 2.8 kb are dimers of monomers 4.1 and 1.4 kb, respectively. Sequence analyzed was dimer 2.8 kb (pGT704-2.8) (GenBank accession: FJ222255). Nucleotide Basic Local Alignment Search Tool result shows that pGT704-2.8 encodes for putative DNA-directed RNAP. This RNAP has sequence identity with RNAP encoded by several mtplasmids found in fungi, namely pHC of *Hebeloma circinans* (75%), pLP of *Pleurotus ostreatus* (74%), pFv of *Flammulina velutipes* (70%), pEM of *Agaricus bitorquis* (59%), pAL2-1 of *Podospora olymera* (55%), *kal* of *Gelasinospora sp.* (54%), *kalilo* of *Neurospora intermedia* (54%), and pCLK of *Claviceps purpurea* (53%).

Nucleotide sequence of pGT704-2.8 was translated using special code for mitochondria. The following is the amino acid sequence of putative DNA-directed RNAP:

MNHRDKYQEYENIYDYDEKSSINSLEANNLHKIDALLVKHVLEVLVD
VITIHCDFGTRIKDVAKLIDVNNIYYQRYTSNDNYSIFILX

The sequences are in conserved domain of RNAP ([Figure 1](#)). The figure shows that RNAP of pGT704-2.8 is a part of single chain RNAP

family and resembles RNAP of T-even phages group (T3/T7). Basic Local Alignment Search Tool protein search reveals that this putative protein has identity with RNAP of other mtplasmids found in *Hebeloma circinans* (41%), *Pleurotus ostreatus* (40%), *Agaricus bitorquis* (39%), *Flammulina velutipes* (39%), *Claviceps purpurea* (32%), *Gelasinospora sp.* (30%), *Neurospora intermedia* (29%), *Podospora anserina* (30%), and RNAP of Phage T3 (53%).

[Figure 2](#) shows the alignment of conserved domain of several fungal RNAPs which has high identity with RNAP found in pGT704-2.8. [Figure 3](#) shows motifs resulted using Multiple EM for Motif Elicitation (MEME) software developed by [Bailey and Elkan \(1994\)](#). Based on this MEME motif, RNAP of pGT704-2.8 does not have solid motif on block X so it cannot be produced by the program, while the block XI can be generated. [Figure 4](#) shows phylogenetic tree reconstructed using Geneious version 8.0.4. RNAP of pGT704-2.8 is in monophyletic group (clade 1) with RNAP of mtplasmids of *Hebeloma circinans*, *Pleurotus ostreatus*, and *Agaricus bitorquis*, all of them belong to division Basidiomycota which have two linear plasmids in their mitochondria. *Flammulina velutipes* also belongs to division Basidiomycota although it is in the same monophyletic group (clade 2) together with *Claviceps purpurea*, *Podospora anserina*, *Gelasinospora sp.*, and *Neurospora intermedia* which belong to division Ascomycota that have one linear plasmid in their mitochondria.

4. Discussion

Linear mtplasmids have often been found in fungi and plants. The first discovery of this kind of plasmid in animal kingdom was in *P. caudatum* (protozoa: ciliates) ([Tallei et al. 2002](#)). Furthermore, the linear mtplasmid was found in *Oxytricha trifallax* (protozoa: ciliates) by [Swart et al. \(2012\)](#). This plasmid has a size of approximately 5 kb and has 251 bp sequence with identity 82% with its mitochondrial genome (mtDNA), so it is suggested that this mtplasmid had been integrated in the mtDNA.

Circular mtplasmid usually has one ORF encodes for one DNA polymerase or one reverse transcriptase, while linear mtplasmid generally has two ORFs encode for putative DNA polymerase and RNAP, in which their amino acid motifs have homology to viral polymerases ([Griffiths 1995](#)). In linear plasmids, DNA polymerase and RNAP each is encoded by one single plasmid, or encoded by different plasmid if there are more than one plasmids in mitochondria ([Cermakian et al. 1997](#)).

According to [Griffiths \(1995\)](#), the characteristics of the linear plasmid among others, have the ORF that encodes a viral type of DNA polymerase and RNAP similar to RNAP of bacteriophage or

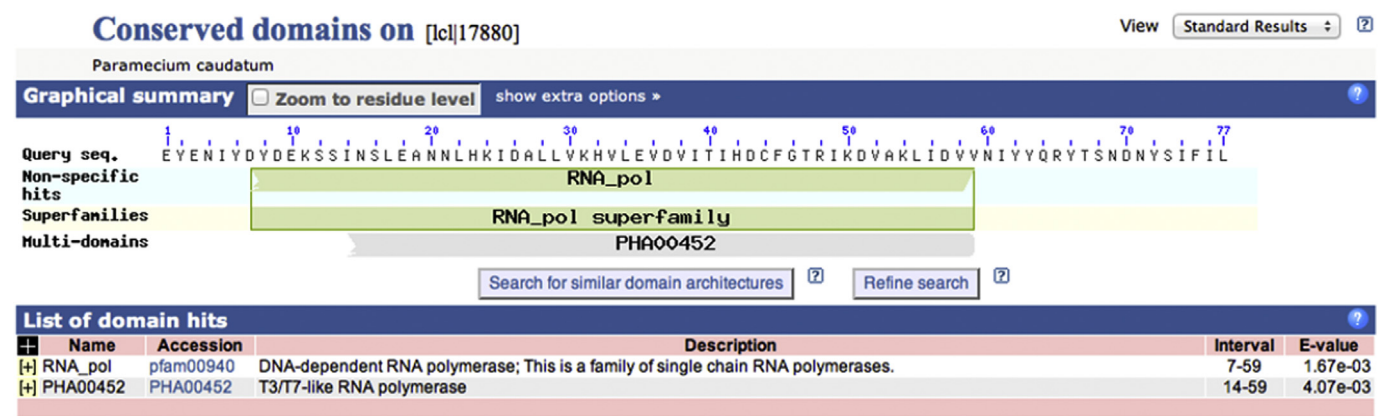


Figure 1. Conserved domain of RNA polymerase (RNAP) of pGT704-2.8 based on conserved domain search developed by [Marchler-Bauer et al. \(2011\)](#) showing that this RNAP belongs to DNA-dependent RNAP, a family of single chain RNAP and resembles T3/T7-like RNAP.

	X										XI									
<i>Paramecium caudatum</i>	EYENIYDYDE	KSSINSLEAM	NLHKIDALLV	KHVLEVL-DV	ITTHDCFGTR	IKDVAKLIDV	V-NIYYQRYT	SNDNYSIFI												
<i>Pleurotus ostreatus</i>	DYSSIKTGLM	PNTIHSLDAS	NIHMLINLII	QLQIKN-MNL	YTHDCFATD	YKNIALLEIL	IKKSPTDIYF	NKK-YLDFI												
<i>Hebeloma circinas</i>	DYPKIKLGLM	PNTIHSLDAS	NIHLLISNIN	KYNLKD-LNL	YTHDCFASD	YKNIAIIEEL	VKHSFIELYF	QVD-YLEAI												
<i>Agaricus bitorquis</i>	DVLSTKRSFM	PNTIHSLDAS	NVHLLNSV-	--SYKN-LPV	YTVHDCFAST	ANNMFKLEKL	VKNAFINIYF	NDEGYLLKL												
<i>Gelasinospora sp.</i>	DSRREVPAII	PNTIHSLDAS	HLPMLI----	DSWDS----Y	ILSIHDCFGT	HPNDMFKLAE	QVRECFILLY	SKND-FLSK												
<i>Neurospora intermedia</i>	DSRREVQAI	PNTIHSLDAS	HLMTII----	DSWDS----Y	ILSIHDCFGT	HPNDMYKLAE	QVRECFILLY	SKND-FLNK												
<i>Flammulina velutipes</i>	NTFKQELAI	PNTIHSLDAS	HLYEIIETR	KNNIN----N	ILSVHDCFGT	HPNKMGLDIH	AVRKTFFIYQ	TDLN-FLNK												
<i>Claviceps purpurea</i>	DKVKQQIALM	PNTIHSLDAS	SL-IMLYFAF	NKSIGSGVVN	FYSVHDCYGV	TAKYIDLLIS	QLRAVYIELY	SKDG-YIAH												
<i>Podospira anserina</i>	NNSKQITAFM	PNTIHSLDAS	SLTLLLDYF	KESID-VKNI	YTHDCFAYP	ANKMECLISL	LKLTYYIKLYS	DDK-YLLKL												
<i>Enterobacteria Ph T3</i>	DAHKQESGIA	PNTVHSQDGS	HLRMTVVYAH	EKYGIE---S	FATIHDSFET	IPADAGKLFK	AVRETMVITY	ENND-VLAD												

Figure 2. Amino acid sequences alignment of pGT704-2.8 RNA polymerase (RNAP) with other fungal RNAPs showing two conserved motifs at blocks X and XI.

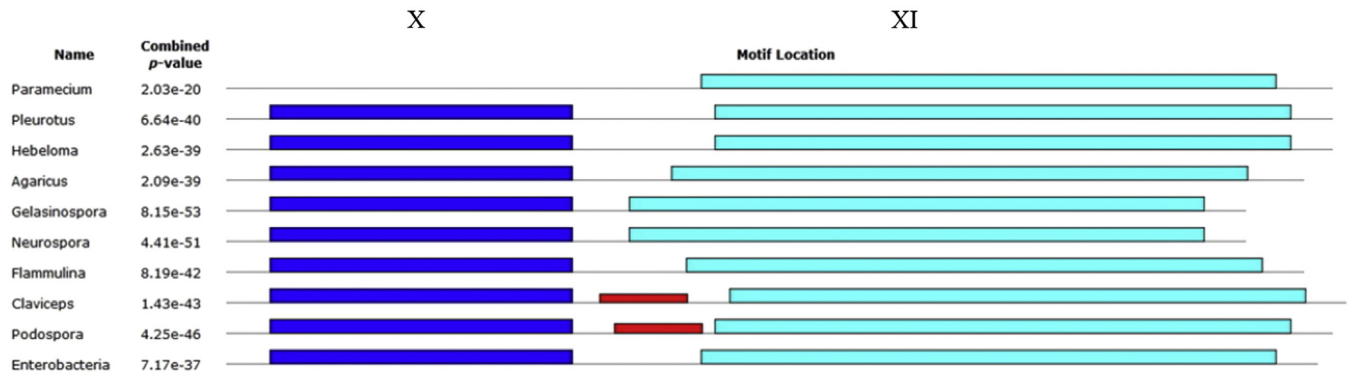


Figure 3. Motifs X and XI generated using MEME motif.

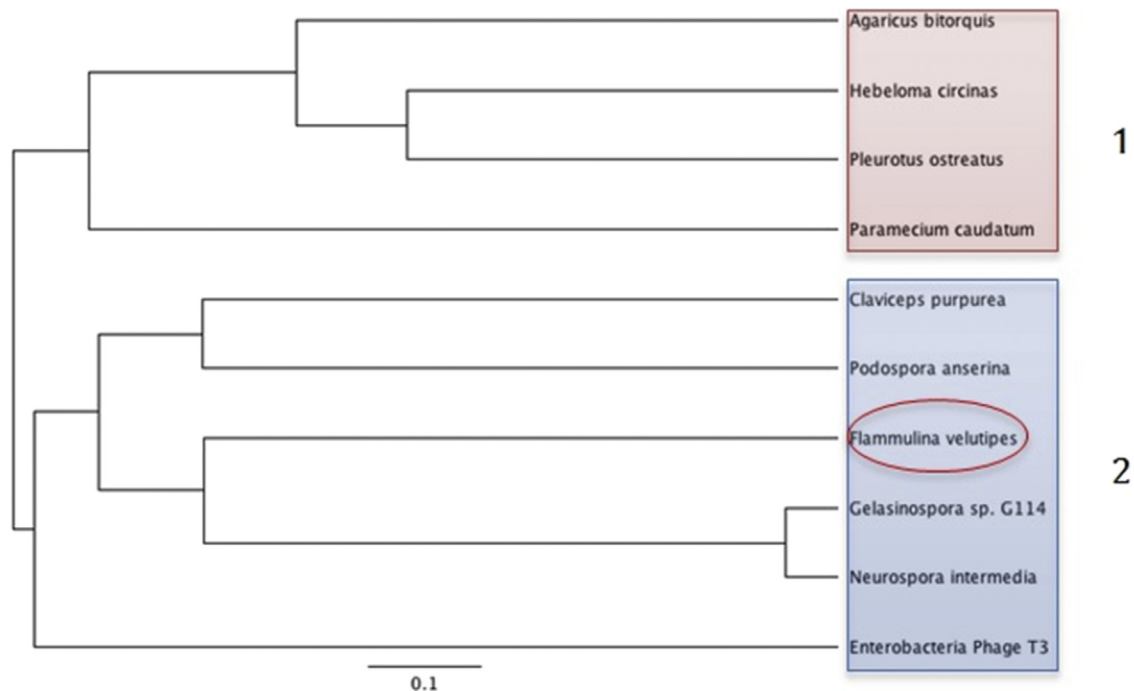


Figure 4. Phylogenetic tree reconstructed using Geneious version 8.0.4 with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method without outgroup. Alignment type is global alignment with free end gaps and cost matrix Blossum 62. Jukes-Cantor was used for genetic distance model. Clade 1 has two linear plasmids and clade 2 has one linear plasmid, except *Flammulina velutipes* which has two linear plasmids. The scale bar corresponds to 0.1 amino acid substitutions per site.

yeast mitochondria. This type of RNAP is also named DNA-dependent RNAP, which is a single polypeptide and does not require any other polymerase for promoter recognition. DNA-dependent RNAP catalyzes the transcription of DNA into RNA by using four ribonucleotide triphosphate as its substrate (Youn *et al.*

2001). RNAP coding gene is probably the gene that undergoes fragmentation because of plasmid integration into mtDNA through recombination. Integrated plasmid sequences have degenerated over time in the absence of selection pressure (Hausner 2003). Plasmid RNAP is presumably bifunctional that recognizes

sequences at the end terminal of plasmid and promoter sequence (Vickery 2011).

Putative RNAP encoded by pGT704-2.8 is homolog to RNAP of T-even bacteriophages and of mtplasmids from divisions Basidiomycota and Ascomycota. Analysis using QuickPhyre (Kelley and Sternberg 2015) shows that RNAP of pGT704-2.8 is located on DNA/RNAP superfamily and on T7 RNAP family with 92.1% confidence by the single highest scoring template. This is supported by Figure 1 which shows conserved domain of RNAP of pGT704-2.8 is based on conserved domain search, suggesting that this RNAP belongs to DNA-dependent RNAP, a family of single chain RNAP and resembles T3/T7-like RNAP. In this group of RNAP, a number of conserved motifs have been identified (Chan et al. 1991). Cermakian et al. (1997) states that motif X (INSLEA: Ile-Asn-Ser-Leu-Glu-Ala in pGT704-2.8) resides in the nucleotides 783–788 while motif XI (IHDCF: Ile-His-Asp-Cys-Phe) resides in the nucleotides 810–814. Considering the length of pGT704-2.8 as 2.8 indicates that the ORF is located in the center. Motif INSLEA is a conserved motif at block X according to Oeser (1988). Motif proposed by Oeser and Tudzynski (1989) for block XI is bHDCF. This matches motif IHDCF found in pGT704-2.8 (Figure 2).

In well characterized RNAP of bacteriophage T7, these motifs were found in a proteolytic fragment which does not have 172 N-terminal amino acid but still retains a catalytic activity. According to Chan et al. (1991), the presence of these motifs (motifs I–XII) indicates that the RNAP is functional. The discovery of only two motifs found in pGT704-2.8 is because the sequencing process is done only partially and randomly. The most conserved part of a protein usually is an important part of the function of the protein. This section is often surrounded by a less conserved structure that modifies the function of the protein. The changes to these additional structures often result in minor changes to the character of protein, for example, the differences in substrate specificity, although the function of the protein is not changed (Černý et al. 2014).

Conserved domain usually is a functional domain, so most likely proteins that are within one family that has the same motif shares the same function as well. Sequence alignment and phylogenetic analysis are aimed at inferring gene function. If the percentage of identity between two compared proteins is >35% (>40% in case of long sequence alignment), it is alleged that the two proteins are homologous, because it has the same structure and function. Homology will decrease when it is in a twilight zone, 20%–35% identity of protein sequence (Rost 1999). Hence, RNAP of pGT704-2.8 can be said to be homologous to the mitochondrial RNAP from plasmid contained in *Hebeloma circinans* (41%), *Pleurotus ostreatus* (40%), *Agaricus bitorquis* (39%), *Flammulina velutipes* (39%), and RNAP of enterobacteria phage T3 (53%).

In the phylogenetic tree (Figure 4), RNAP of pGT704-2.8 is clustered together in a group of RNAP of fungal mtplasmid of *Hebeloma circinans*, *Pleurotus ostreatus*, and *Agaricus bitorquis* which belong to division Basidiomycota with two linear plasmids (clade 1). This is supported by the finding from our previous research (Tallei et al. 2002) that pGT704 has two sets of linear plasmids. *Flammulina velutipes* also has two linear plasmids and belongs to division Basidiomycota although not clustered in clade 1. *Claviceps purpurea*, *Podospora anserina*, *Gelasinospora* sp. and *Neurospora intermedia* belong to division Ascomycota with one linear plasmid. Mitochondrial plasmid pMLPw from *Pleurotus ostreatus* has 5'-end TP. This plasmid has two ORFs, one of them encodes for protein product homolog to RNAP of yeast mitochondria and bacteriophages (Kim et al. 2000). Mtplasmids found in fungi and plants have 5'-end TP and terminal inverted repeat, and encodes for RNAP (Meinhard et al. 1990). Previous research

conducted by Tsukii et al. (1994) revealed that mtplasmid GT704 has covalently bonded protein at 5'-end. Based on this information, it is assumed that pGT704-2.8 shares similar structure.

The similarity between mitochondrial linear plasmid with some phages and viruses implies that they may have originated from a common ancestor, namely virus (Kempken et al. 1992; Griffith 1995). Even though RNAP of pGT704-2.8 has a high identity with enterobacteria phage T3 (53%), but it has a big gap. A gap is a space that is introduced to the process of alignment to compensate for insertions and deletions. RNAP of T7 has N-terminal domain that participates in interaction of RNAP at promoter's specific sites and the binding to the adjacent RNA by changing conformation in the area. At element-1 of AT-rich (adenine -thymine-rich) DNA recognition area (residues 93–101), specificity loop (residues 739–770) and intercalating β -hairpin including Val237 (residues 230–245) hold responsibility for promoter recognition, binding, and melting (Draculic et al. 2014).

The same thing occurred to the function of putative replication protein O of *M. perniciosus*. Although there is lack of information, but with the high identity of its structure and sequence with other RNAP mtplasmids, it was assumed that implication of this enzyme was in the mechanism and expression of mitochondrial genome (Andrade et al. 2009). The enzyme has a length of 766 amino acids and belongs to family of single chain RNAP that is common to virus and cellular organelles (Andrade et al. 2013). The gene of this protein is expressed in almost all phases of fungal life cycle (Andrade et al. 2013).

Replication of bacteriophage T7 DNA is initiated by the binding of T7 RNAP to origin of replication site and production of short RNA molecule that functions as primer (Shi 2013). Carboxyl terminus of phage's enzyme is thought to be involved in promoter recognition, while its catalytic activity is located at terminal end of protein at the downstream region of amino acids 172 (T7) and 173 (T3). Amino-terminal end of T7 RNAP is proposed to be involved in stability of the polymerase-promoter complex (Masters et al. 1987). RNAP of bacteriophages T7/T3 has a simple structure, namely single protein subunit capable of performing transcription cycle without any other protein transcription factor. The rate of synthesis is higher than RNAP in bacteria (Tunitskaya and Kochetkov 2002).

Genes of RNAP, besides having a very important function in the process of transcription and replication, their sequences evolve relatively rapid, so it can be used as a marker in phylogenetic studies. The gene can also be used in the study of DNA barcoding (Singh et al. 2012; Krawczyk and Sawicki 2013; Enan and Ahamed 2014).

This study infers that DNA-dependent RNAP of mtplasmid of *P. caudatum* pGT704-2.8 belongs to the family of single chain RNAP and homolog to RNAP of T-even bacteriophages group, as well as RNAP of mtplasmid from divisions Basidiomycota and Ascomycota. Its function is to perform the transcription without any other protein transcription factor, because the protein is involved in promoter recognition and catalyzing transcription of DNA into RNA using four type of ribonucleotide triphosphate as it substrate. It is also presumed that this RNAP is involved in mtplasmid replication. Considering only the two conserved motifs found in this protein, it could not be ascertained whether the protein has a full function independently or integrated with mtDNA in carrying out its functions.

Conflict of interest

The author declares that there is no conflict of interest attached to this publication.

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